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Visualization of the Transparent, Gelatinous House of the Pelagic Tunicate *Oikopleura vanhoeffeni* Using *Sepia* Ink

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Abstract. Appendicularian tunicates of the genus *Oikopleura* feed using an external, acellular, transparent structure known as the house. Previously, dilute particulate dyes have been used to visualize the internal structure of this house. However, because of toxicity, large particle size, and flocculation, many of these dyes have been of limited practical and scientific use. We report on a new marker, the ink from the cephalopod *Sepia officinalis*, that solves many of these problems.

Specimens of *Oikopleura vanhoeffeni* relished *Sepia* ink, having dark black stomachs and producing many dark fecal pellets over several days. When *O. vanhoeffeni* expanded houses in dilute ink, the internal walls, septae, and filters were shown in great detail, whereas high concentrations of ink showed delicate patterns of lines on the internal walls.

We present documentary photographs of previously unillustrated or undescribed morphologies: the escape slot; the incurrent funnels; two dimples caused by insertion of suspensory filaments on the upper wall of the posterior chamber, a large, posterior keel; both the open and closed positions of the exit valve; and the complex pattern of lines on the inner walls. However, the external walls of the house had no affinity for the dye and could only be seen by dark field illumination.

We believe that *Sepia* ink can be used to visualize functionally important transparent structures of other gelatinous zooplankton and can be a colloidal marker in feeding experiments of a wide range of filter feeders.

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Introduction

Oikopleurid appendicularians are suspension feeding zooplankters that are surrounded by a transparent, acellular, gelatinous "house," which they secrete. The house contains a complex system of fine filters that are used by the animals to concentrate and remove food particles from suspension. Using its muscular tail as a pump, the animal draws water into the house through a pair of coarse, bilateral, incurrent filters. The water is then pumped through the tail chamber into bilateral passageways leading to the lateral edge of expansive food-concentrating filters. Here much of the water is pushed through a mesh with 0.22 μm pore size (Deibel *et al.*, 1985). Particles are retained between the food concentrating filter screens, resulting in a concentrated food suspension that is drawn into a medial food-collecting tube leading to the animal's mouth. This food suspension is 100 to 1000 times more concentrated than are particles in the environment surrounding the animal (Jörgensen, 1984; Flood, in prep.). A third filter inside the pharynx of the animal traps the food particles for ingestion. The filtered water exiting the food concentrating filter leaves the house through a narrow exit spout and valve, producing a jet that propels the house and animal slowly through the sea.

The existence of the house has been known since the work of Fol (1872) and Lohmann (1899). However, many details of its structure remained unknown until recent improvements in microscopical techniques and special staining procedures made further progress possible. Dilute particulate dyes have been used to visualize the internal walls, chambers, and filters of the house (Alldredge, 1977; Flood, 1978, 1983; Deibel *et al.*, 1985;

Deibel, 1986; Fenaux, 1986). When added to seawater, dye particles are retained by the filters within the house in the same way as are naturally occurring particles. Lohmann (1899) and Alldredge (1977) used dilute suspensions of carmine particles to visualize both the incurrent and food-concentrating filters of many oikopleurids. However, the animals may not feed normally when carmine is present (Alldredge, 1977). We have found that freshly prepared dilutions of carmine and extreme care are required to prevent the animals from leaving their houses. In addition, carmine particles settle rapidly and stain only the incurrent and food-concentrating filters.

Fenaux (1986) used dilute India ink to stain the incurrent and food concentrating filters, and the internal walls and septae of houses of *Oikopleura dioica*. One of us (P.R.F.) has used a similar technique since 1978. If India ink is added to seawater before the animal expands a new house, all internal walls and septae of the house are stained (Flood, in prep.). However, if the ink is added after the house has been expanded, only the food-concentrating filter is stained. This approach requires freshly prepared ink solutions and great care to prevent the animal from leaving its house. The carbon particles that make up India ink tend to aggregate in seawater and settle rapidly as do particles in carmine suspensions.

Deibel (1986) used several types of particles to mark specific parts of the house of *Oikopleura vanhoeffeni* differentially. These particles included finely ground charcoal, starch, latex beads, and the unicellular green alga *Isochrysis galbana*. Charcoal particles adhered specifically to an intermediate, coarse screen between the two walls of the food-concentrating filter. The alga, on the other hand, stuck to the upper and lower walls of the food-concentrating filter. Starch granules did not adhere to any of the filters of the house, but stained the pharyngeal filter within the trunk of the animal. This suggests that physical and chemical properties of both the marker and the house structures in question affect the staining result.

We recently found another marker that may be used to visualize structural details of oikopleurid houses that, in the past, have been difficult or impossible to document. Information about these structures is needed to understand the behavioral and functional details of the feeding process on which the ecology of these animals depends. The new marker may be used not only to visualize feeding structures and quantify particle clearance rates of pelagic tunicates, but also to observe transparent structures of other marine plankton.

Materials and Methods

Individuals of *Oikopleura vanhoeffeni*, in their houses, were collected in 500-ml glass jars by SCUBA diving in Logy Bay, Newfoundland, during May and June 1989. Animals were maintained in these jars for up to 10 days in laboratory tanks containing circulating seawater at 1 to 5°C, about 1°C above ambient sea surface temperature.

Ink was collected from freshly dead specimens of the cuttlefish *Sepia officinalis* at the Plymouth Marine Laboratory, Devon, U.K. The ink duct of each animal was clamped with a hemostat while the ink sac was removed. Once excised, a loop of the duct was placed in a collection vial and the duct cut to allow the ink to drain into the vial. This ink was diluted immediately with 10 parts of distilled water and 10,000 units of pennicillin G added per ml of solution to help prevent bacterial decomposition. Diluted ink remains liquid for several years, but undiluted ink coagulates after several weeks at room temperature. The ink was dispersed with gentle agitation in seawater to a dilution of *ca.* 10^{-5} just before use.

Solid *Sepia* ink is available commercially, but must be ground before use. It also contains phenol or other chemical preservatives that may be noxious or toxic to marine animals, and therefore it was not used in these experiments.

Houses were examined using a Wild M420 macroscope with bright or dark-field illumination. The light source used for routine observation was a 100W halogen lamp, whereas two modified Sunpak GX14 electronic flashes and a Wild MPS 55/-51 photoautomat were used for still photography. We used Kodak Ektachrome 100 and 400 ASA film for slides, and Kodak Tmax at 100 to 3200 ASA for prints. By using 0.5- and 2.0-times accessory lenses on the macroscope, final magnification of the photographs ranged from 1.25- to 25-times.

Results and Comments

Liquid *Sepia* ink was easily miscible in seawater, and, contrary to other dyes that have been used to visualize the house of Appendicularia, it stayed evenly dispersed in solution for up to 14 days.

Transmission electron microscopy (TEM) revealed that this ink consists of uniformly spherical melanin granules with diameters ranging between 56 and 161 nm (arithmetic mean = 102 nm, Standard deviation = 21 nm, Flood, pers. obs. by TEM).

In spite of this low particle size, some of the ink particles were easily concentrated and ingested by *Oikopleura vanhoeffeni*. In fact, these animals seemed to relish the *Sepia* ink, having full stomachs and producing abundant opaque, black fecal pellets (Fig. 1) that appeared to be composed entirely of ink.

However, much of the ink passed through the house of *Oikopleura vanhoeffeni*, without being witheld by the food-concentrating filters. Some of these particles ad-

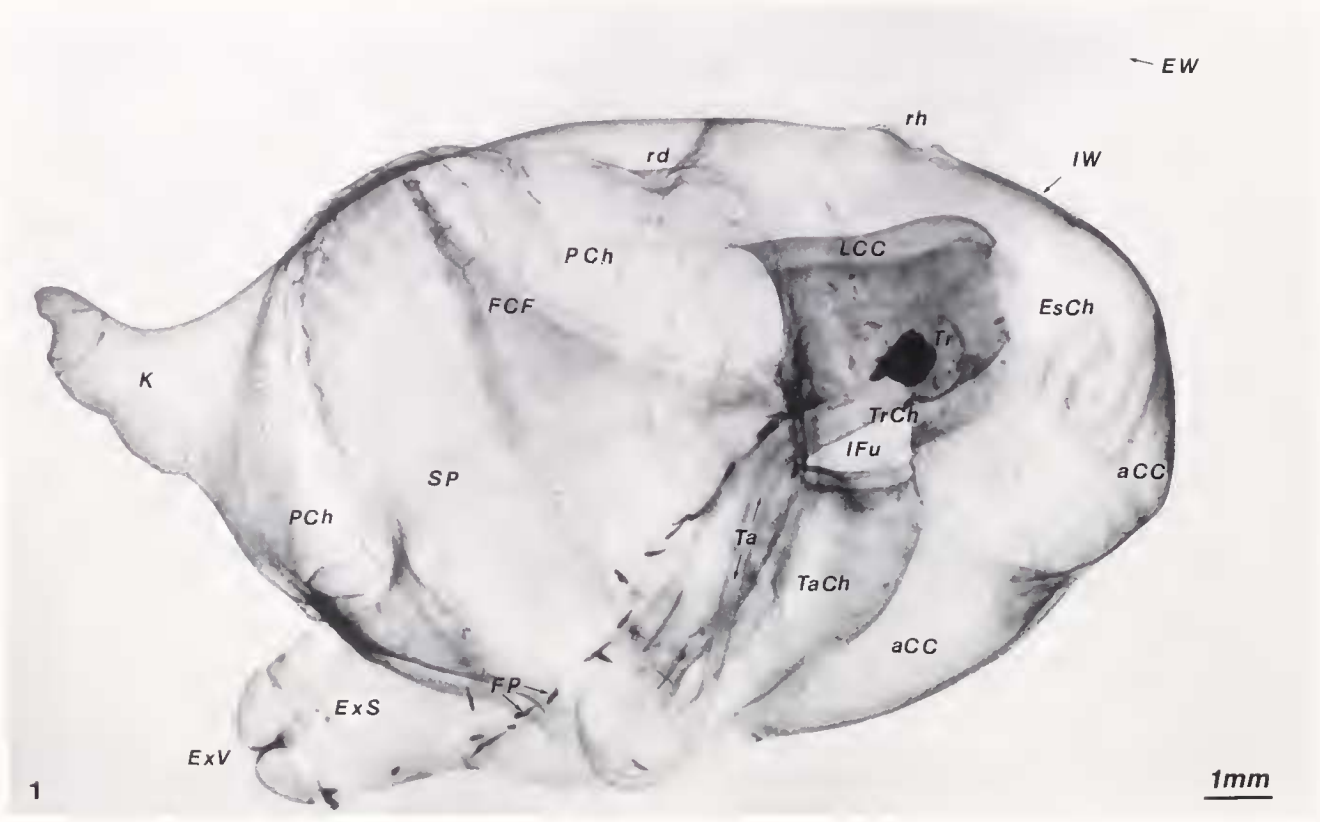


Figure 1. Lateral view of a live *Oikopleura vanhoeffeni* beating its tail inside a house faintly stained by *Sepia* ink. Bright field macrograph at seven times magnification. [The nomenclature used is adopted from Flood (1983) and is largely a direct translation of Lohmann's German names (Lohmann, 1956).]

In addition to numerous details of the inside walls (*IW*) of the house, like the prominent exit spout (*ExS*) and valve (*ExV*), a keel (*K*), cushion chambers lateral (*LCC*) and antero-medial (*aCC*) to the inlet openings, inlet funnels (*IFu*), roof dimples (*rd*), and a roof hump (*rh*), numerous internal details can be seen. The animals trunk (*Tr*), tail (*Ta*), and escape chamber (*EsCh*) as well as the trunk chamber (*TrCh*), tail chamber (*TaCh*), supply passage (*SP*), and suspension of the food-concentrating filters (*FCF*) in the posterior chamber (*PCh*) are faintly outlined. Numerous fecal pellets (*FP*) stained completely black by *Sepia* ink are seen along the floor of the posterior chamber. The external wall (*EW*) is only visible above the hump in the inside roof.

hered to the internal walls and septae of the house and made them easily visible.

By varying the concentration of *Sepia* ink from experiment to experiment, the intensity of staining could be controlled to reveal different features of the house. When a specimen of *Oikopleura vanhoeffeni* expanded its house in seawater containing very dilute *Sepia* ink, the internal walls, septae, and filters were shown in great detail (Fig. 1), whereas heavy staining made the house less transparent and revealed delicate patterns of lines and fields on the internal walls and septae throughout the house (Figs. 2, 3).

The outer wall of the house, however, had no affinity for the ink and was rarely seen at all in bright field illumination (Fig. 1). However, in most cases its presence was revealed by adhering detritus particles. This was particularly true for the prominent bow of the house (Fig. 2). In dark field illumination, on the other hand, the external walls and their variable thickness in distinct parts of the house became more evident (Fig. 3).

The difference in volume between the internal water-filled spaces and the total house could be estimated from such pictures. If the internal transverse diameter of the house was considered to be unity, the external transverse diameter was generally close to 1.2, the internal longitudinal diameter about 1.3, and the outside longitudinal diameter about 1.7. Considering the house to be an elliptical rotatory body, this makes the total volume approximately 1.5 times as large as the internal water-filled spaces. We do not know if the spaces between the inside and outside walls are filled with a compact (gel-like) substance or if they are water-filled chambers unaccessible to the *Sepia* particles.

By varying the staining intensity of the house, we dis-

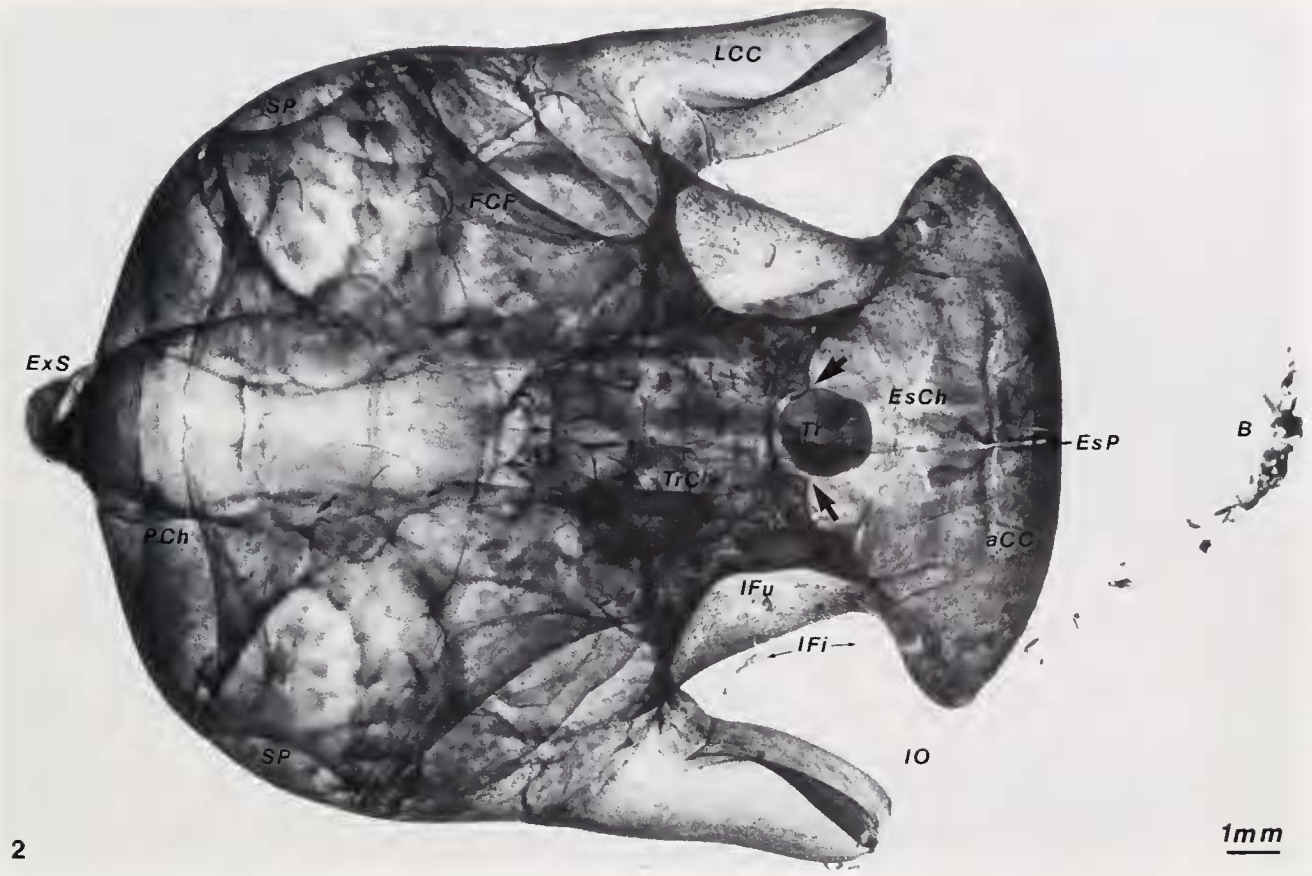


Figure 2. Top view of live *Oikopleura vanhoeffeni* inside its house. Bright field macrograph at seven times magnification after strong staining with *Sepia* ink.

Intricate patterns of *Sepia* ink are seen on many walls, as for example near the escape passage (*EsP*) and supply passages (*SP*). Note also the attachment (*arrows*) of the animal trunk (*Tr*) to the walls separating the trunk chamber (*TrCh*) from the escape chamber (*EsCh*). The inlet openings (*IO*), inlet filters (*IFi*), and the inlet funnels (*IFu*) are visible on both sides of the house. Note the prominent bow (*B*) made visible only by adhering detritus particles. Otherwise, same labeling as in Figure 1.

covered many structural details of which we were previously unaware or had insufficient knowledge. Here we will only describe some of the most prominent features and comment briefly on their functional significance.

(1) *The escape port in the anterior chamber (Figs. 2, 5B).* The animal forces its way through this preformed weak part when it leaves the house, thereby tearing it open to a wide escape slot. This escape port is covered by the massive bow of the house (Fig. 2), and somehow a preformed channel must exist through this bow material towards the external house wall. Otherwise the animal could not force its way out of the house as easily, frequently, and uniformly as it does (*cp.* Fenaux, 1985).

(2) *The incurrent funnels leading into the house (Figs. 1–3).* In the only existing description of the house of *Oikopleura vanhoeffeni* (Deibel, 1986, Fig. 1), these have been given quite a different shape from what we have been able to photograph.

(3) *The attachment of the anterior walls of the incurrent funnels to the lateral part of the trunk (Fig. 2).* These walls seem to meet the trunk exactly where the Langerhans bristle is located. Through this sensory organ the animal may monitor accordingly the inflation and condition of the house (Bone and Ryan, 1979). Perhaps the entire house in this context may be regarded as a tactile sensory structure.

A rather rigid suspension of the trunk of the animal within the house is needed for the tail to perform its pumping action. This prevents the "tail from wagging the dog" as may be observed just before the animal leaves its house, when the trunk has detached from some of its anchoring points. Stimulation of the Langerhans receptor may then initiate the vigorous jerk and swimming movement that enables the animal to detach completely from the house and force its way through the escape slot.

(4) *The shape of prominent chambers and lateral flaps*

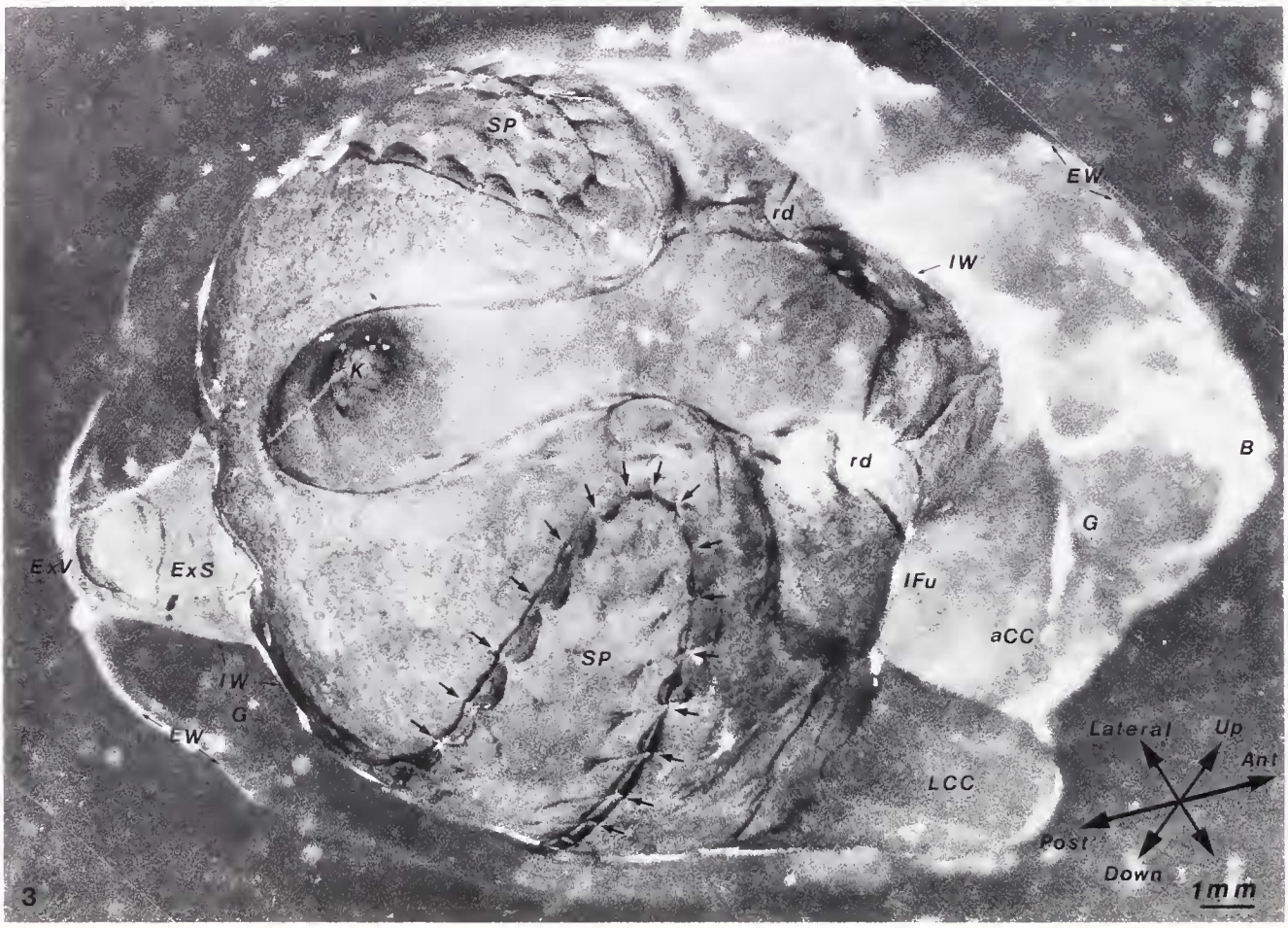


Figure 3. Inhabitated house of *Oikopleura vanhoeffeni* as seen in dark field illumination from a point above, behind, and to the side of the house. (The axes of the house as it normally moves through the sea are indicated in the lower right hand corner.) Magnified seven times.

Note the prominent patterning of the internal walls (*IW*) corresponding to the attachment sites of the filter ridges of the food-concentrating filters (*arrows*) along the periphery of the supply passages (*SP*), and the presence of a prominent semitransparent jelly-like substance (*G*) covering the posterior side and the anterior bow-like pole (*B*) of the house. The outer limit of this jelly-like substance represent the true external walls (*EW*) of the house. The orientation of the house as it moves through the water is indicated by axes in the lower right hand corner of the figure. For other abbreviations refer to Figure 1.

in the anterior part of the house, medial and lateral to the incurrent openings (Figs. 1–3). These chambers are probably filled by water flowing from the tail chamber through a hole in its distal floor (Flood, in prep.). It is also possible that the anterior chambers communicate with the upper compartment of the posterior chamber and may be filled by water via this route, as suggested by Fenaux (1986). A positive pressure in the anterior chambers surrounding the incurrent funnels is needed to resist the collapsing force generated by the negative pressure within these passageways as water is drawn into the house. The lateral flaps may serve as vertical stabilizers to control the orientation of the house as it moves through the sea or as flaps to prevent the immediate reclogging of the incurrent filters after they have been backwashed (Flood, in press).

(5) *Two large dimples in the inner house wall of the upper compartment of the posterior chamber (Figs. 1, 3).* These probably represent the anchoring sites of suspensory filaments originating somewhere along the anterior edge of the food-concentrating filters.

(6) *A medial hump in the inner house wall above the trunk of the animal (Fig. 1).* The external house wall had its highest optical density and could be faintly seen even in bright field illumination above this hump. Although of unknown functional significance, this hump is also found in houses of *Oikopleura dioica* and *O. labradoriensis* (Flood, pers. obs.).

(7) *A large "keel" at the back of the house just above the exit valve (Figs. 1, 3).* This keel may serve as a rudder to inhibit rolling and to facilitate looping motions as the house is propelled through the water. A looping motion, which has been described for other oikopleurans by Alldredge (1976), allows the animal to stay within and exploit a patch of nanoplankton more efficiently than by a linear motion. This keel was discovered by Deibel (1986), but due to poor visibility, even in dark field illumination, his description is incomplete (Compare his Fig. 1 to our Fig. 1).

(8) *A posterior exit spout and valve below the longitudinal midline of the house (Figs. 1, 3).* Strong staining by *Sepia* ink allowed us to observe the opening and closing action of this pressure sensitive valve. In its closed position its upper and lower lips were inverted (Fig. 4A). One to five seconds after the pumping action of the tail started and increased the pressure inside the posterior chamber and exit spout, the lips everted and exposed a medial oval opening with a strongly birefringent and elastic rim (Fig. 4B). This central exit opening was evident even when the animal pumped slowly. However, when the tail pumped at maximum efficiency, the exit spout became much longer, and four additional exit openings were exposed peripheral to the central one. The tissue surrounding the exit valves was then stretched to such a degree that it left very little contrast in our photographs (Fig. 4C). Fenaux (1986), studying *Oikopleura dioica* houses, found the four peripheral openings to open before the central one.

The propulsive thrust generated by the jet of water leaving the house was directed somewhat below the center of the house, resulting in a tendency to turn the front of the house upward. When combined with the slightly upward-pointing bow and the directional control of the keel and lateral flaps (see above), this thrust will result in a slow upward movement of the house, or even a vertical looping motion as sometimes seen in the field (*cp.* Alldredge, 1976).

The more intense staining resulting from higher concentrations of *Sepia* ink revealed delicate patterns of lines and fields on most internal walls of the house (Figs. 2, 5). In some areas, complex patterns of straight or curved lines were visible (Fig. 5A). These may correspond to decorated filaments, corrugated surfaces, or small pockets. In other areas, faint patterns of polygonal fields were apparent (Fig. 5B). Although each polygon was quite large, their pattern reminded us of the oikoplast cell pattern on the trunk of the animal. These cells are responsible for the production of the house (Lohmann, 1933/1956); perhaps *Sepia* ink might be used to map the areas of the house made by individual cells. This represents a major problem yet to be properly elucidated for all appendicularians.

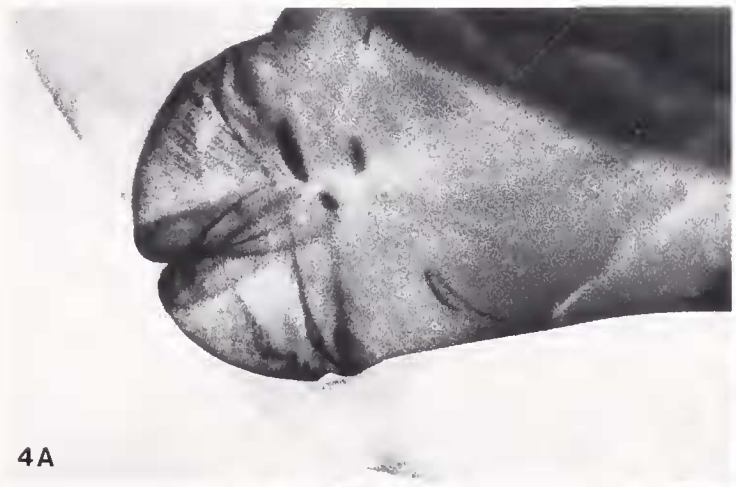
Discussion

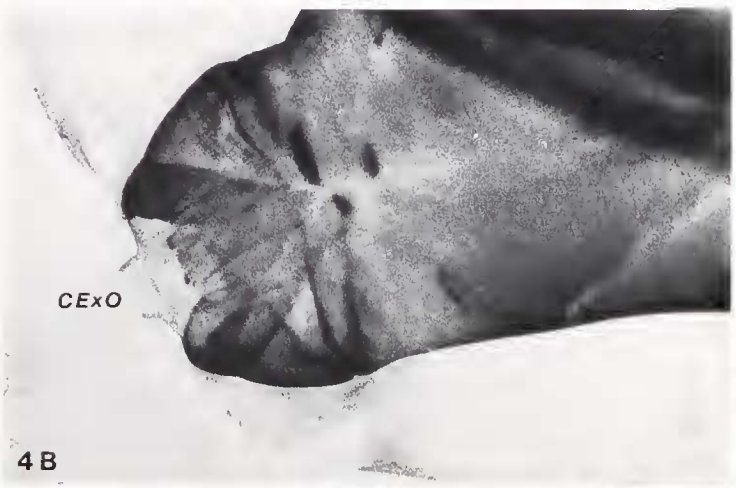
The usefulness of the *Sepia* ink for visualizing distinct parts of *Oikopleura* houses probably depends on three or four factors: (1) *Sepia* ink forms stable solutions in seawater and does not aggregate and sediment like most other particulate dyes. Such flocculent particles seem to interfere with the house expansion process of animals kept in captivity. (2) The particle size of *Sepia* ink is small enough to allow a significant proportion of particles to pass the food-concentrating filters to stain the walls of the posterior chamber, the exit spout, and possibly the anterior chambers of the house. (3) The animals seem to relish the *Sepia* ink as a food source and do not find it noxious or toxic like many other dyes. (4) The physico-chemical properties of the *Sepia* ink particles may be particularly favorable to stain the internal walls and septae of the house.

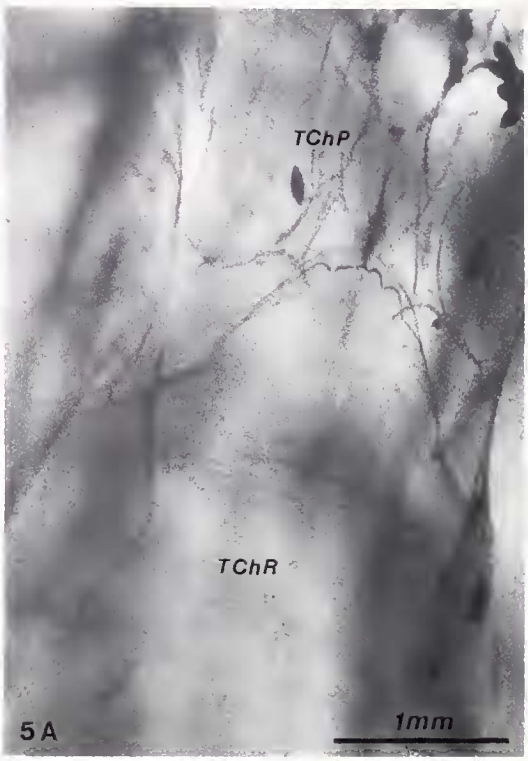
These excellent properties of *Sepia* ink may make it useful in the study of other gelatinous zooplankters.

The reason why *Sepia* ink, like all other particulate dyes we have used, failed to stain the external walls of the house remains obscure. It may depend on special physico-chemical properties of this layer, but a more likely explanation may be that the dye particles are prevented from having direct physical contact with it. The walls surrounding the water-filled spaces inside the house are probably not entirely waterproof. Due to the higher hydrostatic pressure inside the house, water will seep slowly out through the walls, leaving its particles behind as a decoration on the internal walls, and producing a thin halo of particle-free water just outside the house. Such a halo may be enough to prevent the proper staining of the external walls.

The pore size of the food-concentrating filters of *Oikopleura vanhoeffeni*—1.0×0.22 μm according to Deibel *et al.* (1985)—was significantly larger than was the particle size of *Sepia* ink (0.1 ± 0.02 μm according to Flood, unpub. res.). In spite of this, the animals used in this study easily concentrated and ingested the dye, and incorporated it into fecal pellets. This may depend on a selection of the largest particles in the ink, on a selection of aggregated particles, or on an ability to retain smaller particles than hitherto believed. In fact, the carbon budgets of oikopleurans seem to be such that ingested particles > 0.2 μm in diameter rarely account for more than 30% of the energy expenditure for growth, respiration, and house production (Paffenhofer, 1976; Gorsky, 1980; King, 1981). It seems likely that the animals may obtain much of their nurishment from particles < 0.2 μm in diameter, or from dissolved organic matter. We foresee the use of monodisperse *Sepia* ink particles in future feeding experiments on appendicularians and other filter feeding marine animals.







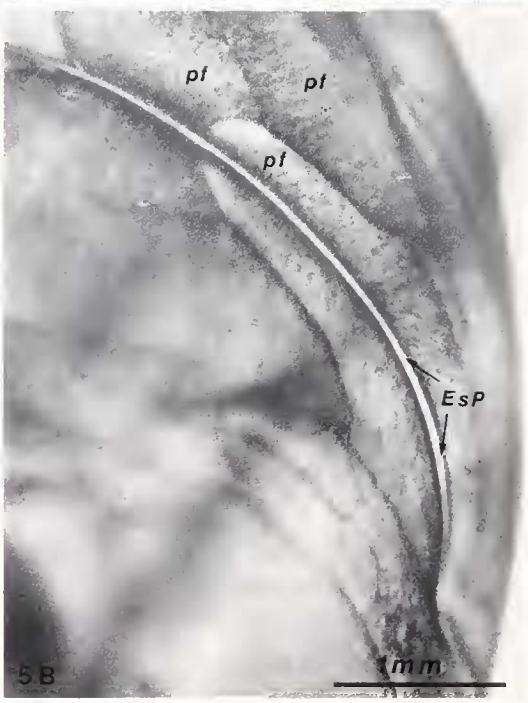


Figure 4. Bright field macrographic details of the exit spout and valve of a heavily *Sepia*-ink stained house of *Oikopleura vanhoeffeni* at 20 times magnification.

(A) In its closed state, (B) in its half open state, and (C) in its full open position. Unfortunately the exit openings themselves [one central (*CExO*) and four peripheral (*PExO*)] didn't give sufficient contrast to be seen in picture C. The external wall (*EW*) of the house is seen next to the exit spout.

Figure 5. Bright field macrographic details of an *Oikopleura vanhoeffeni* house heavily stained by *Sepia* ink at 23 times magnification.

In (A), parallel ruffles (*TChR*) and numerous pockets (*TChP*) are seen in the roof of the tail chamber. In (B), polygonal fields (*pf*) resembling cell outlines are seen next to the escape passage (*EsP*) of the house.

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Note added in proof: We have used commercial ink from *Sepia* recently available from Sigma Chemical Co. (St. Louis, Missouri). *Oikopleura vanhoeffeni* took up this ink similarly to that we collected from *Sepia* ourselves.

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